INHIBITION OF TFEB ACTIVATION PROMOTES *COXIELLA BURNETII* GROWTH

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"If you take out the team in teamwork, it's just work. Now who wants that?"

Matthew Woodring Stover

This work of mine is a team effort, its strength lies in its members. I would like to thank Dr. Stacey Gilk, my mentor for her supervision, encouragement, and guidance throughout the course of my work.

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Piya Das Ghatak

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Coxiella burnetii is the etiologic agent of Q fever, a zoonotic disease characterized by flu-like sickness in acute cases; endocarditis may occur and turn deadly if not treated correctly in chronic patients.

Coxiella, an obligate intracellular bacterium, requires establishment of a replicative niche in the host cell. After being phagocytosed by the eukaryotic cell, the bacterium resides in a tight-fitting nascent phagosome which matures through the host canonical endocytic pathway, acquiring endosomal/lysosomal markers as well as acidic pH. Initial acidification of the *Coxiella* containing vacuole (CCV) is central to the bacterium's pathogenesis because translocation of bacterial effector proteins into the host cell by the type 4B secretion system (T4BSS) initiates only after it senses the acidic environment. The effector proteins are required for subverting different host cell functions in favor of *Coxiella* growth, CCV maturation and are crucial for bacterial virulence.

Contrary to the belief that since CCV matures through the host endocytic pathway, CCV is as acidic as lysosome, we found that CCV is significantly less acidic (pH~5.2) than lysosomes (pH~4.8) and inducing further CCV acidification causes *Coxiella* lysis. Furthermore, increasing lysosomal biogenesis in the host cell is detrimental for *Coxiella* growth. So, we hypothesized that *Coxiella* blocks lysosomal biogenesis in host cells to maintain the CCV pH just optimal for its growth.

Lysosomal biogenesis is regulated by the master transcription factor EB (TFEB). Its ability to act as a transcription factor depends on its subcellular localization, which

relies on its phosphorylation state. TFEB, when phosphorylated is cytosolic and inactive, whereas dephosphorylated TFEB translocates to the nucleus and is active, binding to promoter regions of lysosomal genes of the CLEAR network, thus controlling lysosome biogenesis. Therefore, we hypothesized that *Coxiella* blocks TFEB translocation to the nucleus, thus inhibiting lysosome biogenesis.

We determined that *Coxiella* grows significantly better in TFEB-KO cells than they do in parentals. Also, using a torin-induced TFEB translocation model, we observed remarkably decreased TFEB activation in the *Coxiella* infected cells as was evident by less TFEB translocation to nucleus. Overall, data obtained from this work suggest that *Coxiella* inhibits lysosome biogenesis by blocking TFEB nuclear translocation.

Stacey D. Gilk, PhD, Chair

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ACCM2	Acidified cysteine citrate medium 2
CCV	*Coxiella* containing vacuole
CDC	Centers for Disease Control and Prevention
CLEAR	Coordinated Lysosomal Expression And Regulation
LCV	Large cell variant
LPS	Lipopolysaccharide
mTOR	mechanistic target of rapamycin
SCV	Small cell variant
T4BSS	Type 4B secretion system
LAMP1	Lysosome-associated membrane protein 1
DAPI	4', 6'-diamidino-2 phenylindole
CFU	Colony forming unit
TFEB	Transcription factor EB

CHAPTER ONE: INTRODUCTION

History of Q fever: *Coxiella burnetii*

A mystifying disease first reported in 1935 among a small number of slaughterhouse workers in Queensland, Australia, was initially called abattoir fever, as it was consistently prevalent among individuals working with butchering animals at an abattoir [1, 2]. The key characteristic feature of the disease was the incapacitating high fever accompanied by a headache lasting up to two weeks. These symptoms were often presented with pulmonary and influenza-like complications. Since the organism responsible was unidentified for a long time, the disease was renamed query fever (Q fever) [3]. While investigating the disease, Australian researcher Edward Derrick, pathologist in charge at Royal Women's and Brisbane Hospital, was able to successfully infect guinea pigs with blood from infected patients. He sent emulsions of infected guinea pig livers to Frank McFarlane Burnet in Victoria, who was able to isolate the pathogen and hypothesized it to be of rickettsial origin [4]. His experiments infecting a variety of different animal species with the guinea pig liver emulsion revealed pathogenicity and virulence information of the organism in different hosts. By employing new staining techniques, it was determined that the pathogen was "filter-passing" after all; with the ability of surviving in the chorio-allantois of developing eggs without inducing lesions [5, 6].

Around the same time, thousands of miles away in the Rocky Mountain Laboratory in Hamilton, Montana, in the United States, researchers Herald Cox and Gordon Davis were attempting to identify the causative agent of Rocky Mountain Spotted Fever from ticks collected at Nine Mile Creek, Montana. This pathogen was named

"Nine Mile" and caused febrile illness in guinea pigs infected with blood from diseased animals [5]. The first known human transmission case occurred during this time, when Rolla Dyer, the future NIH Director, was infected after working with Cox, who became infected himself while managing contaminated samples. A series of seminal immunological and cross-protection studies demonstrated that guinea pigs which recovered from disease caused by Dr. Dyer's samples were immune to infection with the Australian Q fever agent, thus indicating that the Australian Q fever agent and organism isolated from ticks in Montana were the same organism [7].

The pathogen underwent several name changes over the course of about 80 years since its first report. Starting from being assumed it was a virus by Derrick, to briefly acquiring the names *Rickettsia diaporica*, followed by *Rickettsia burnetii.* Eventually the name *Coxiella burnetii* was given to honor both research groups in United States (Herald Cox) and Australia (Frank McFarlane Burnet) [8, 9].

Human and animal disease

The disease caused by *C. burnetii* is described as clinically polymorphic, as it can manifest into a range of varying forms or types within a single affected species [4]. The main reservoirs for *C. burnetii* are farm animals, such as cattle, sheep, and goats. However, studies in recent years indicate that a significant number of domestic and marine mammals, as well as reptiles, birds and ticks can also shed the bacteria [10]. Regardless of the source, human transmission is primarily achieved by inhalation of bacteria-contaminated aerosols spread in the environment. Moreover, some interesting reports of infection by tick bites, consumption of milk from contaminated animals, and

nosocomial transmission between humans have also been recorded as potential source of infection [11-13]. Thus, Q fever is classified as a zoonotic disease.

In animals, Q fever is mostly asymptomatic, thus making identification of infected animals is challenging. Usually, a conclusive test to determine serum antibody titers is required to determine the infection. Abortions at the final gestational phase in pregnant goats and sheep is the key clinical manifestation of the disease [14]. Up to 90% of infected cattle face abortions, which results in bacterial shedding as high as 10^9/gram placenta into the environment [3, 15]. Urine, feces, and milk from these animals also contribute to the contaminated aerosol load in the environment.

Since *C. burnetii* can resist drying, heat, osmotic pressures, and common disinfectants, it is environmentally a very stable pathogen and the contaminated aerosol particles can survive and persist for a prolonged period in soil and cause further infection in humans. *Coxiella* has the lowest bacterial infectious dose known; fewer than 10 bacteria (ID_{50} = 1) are capable of causing disease [16]. Combined with environmental stability and a low infectious dose, *C. burnetii* fulfills most of the requirements for a biowarfare weapon. Before the United States ended its biological warfare program in 1969, *C. burnetii* was one of the seven pathogens being pursued as a biological weapon [17]. It is currently listed as a category B biological warfare agent by the Centers for Disease Control and Prevention (CDC) [18].

An earlier 1950 study with 51 human volunteers indicated that most efficient route of human infection was intradermal inoculation, which resulted in 100% seroconversion, followed by infected food consumption and intranasal inoculation [19]. However, aerosol transmission remains the most common route of infection probably

because pasteurization of food decreases the probability of infection by the digestive route.

Although the prevalence of cases varies from one country to another, Q fever has been reported across the world, except in New Zealand [15, 20, 21]. However, global Q fever incidence and prevalence rates are often poorly characterized due to varying reportable symptoms and misdiagnoses.

Wide state to state variation of Q fever seroprevalence in cattle has been reported in the United States, but *Coxiella* infection has increased in U.S. farm animals during last few decades [22]. Despite this rising prevalence of Q fever in cattle, reported cases of human infection in the U.S. have been low (Figure 1). According to the most recent report from Centers for Disease Control and Prevention (CDC) in 2017, more than one-third of cases (38%) are reported from states where ranching and raising livestock are common. Seasonal trends of Q fever emergence in the country also follows this trend, most reported cases of illness begin in spring and early summer months which is the birthing season for cattle, sheep and goats [23].

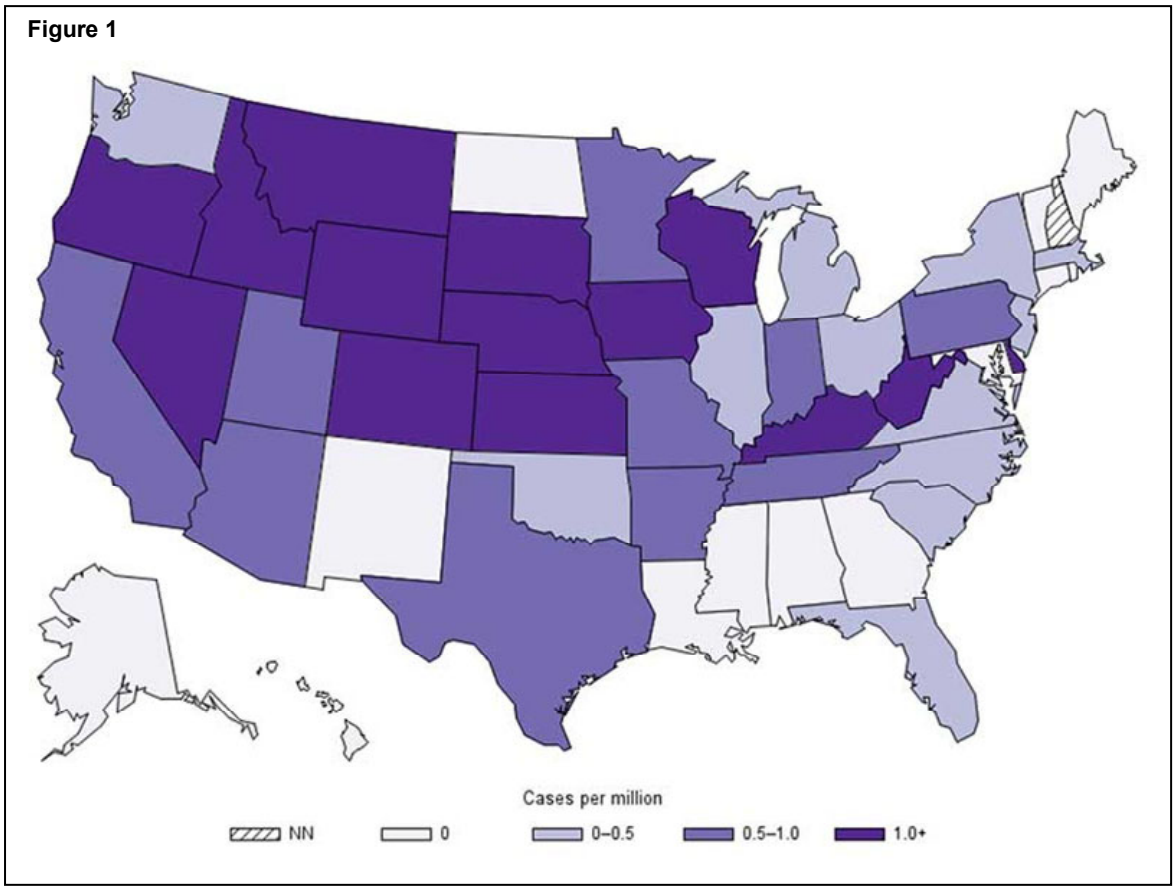

Figure 1: Annual reported incidence (per million people) for Q fever – United States for 2017

In the U. S., Q fever was declared a nationally notifiable disease in 1999. Cases reported by state and local health departments are compiled by the CDC to determine national trends. Passive surveillance systems for Q fever, compounded with lack of reporting and timely diagnosis due to nonspecific presentation are thought to contribute to the underrepresented cases. (NN = Not notifiable)

In humans, the presentation of Q fever can range from asymptomatic to mildly symptomatic seroconversion, which can develop throughout the acute phase of the disease, eventually leading to the life-threatening chronic illness. About 60% of human infections are asymptomatic [24]. In the majority of infections of otherwise healthy individuals, acute disease presents as a self-limiting febrile illness. Symptoms start as flu-like associated with pneumonia. In chronic disease, which develops in approximately 1-2% of acutely infected cases, endocarditis is the major disease outcome, especially in patients with previous valvulopathy. Host factors, such as age, gender and preexisting medical conditions can greatly influence disease symptoms [25].

In addition to Q fever, Q fever fatigue syndrome (QFS) is a serious clinical manifestation of the disease. It is described and well documented in many countries and characterized by persistent fatigue condition, which can last between five and ten years in 10%-15% of the acutely infected population [26]. Though the cause of development of this condition is unknown, lack of satisfactory outcome with existent treatment strategies is believed to be a critical factor [27].

About 370 Q fever outbreaks in different countries have been reported worldwide since 1954. Countries such as Australia, USA, Germany, France, The Netherlands, and United Kingdom have reported small-scale Q fever outbreak notifications at different times. However, the largest Q fever outbreak recorded to date occurred in the Netherlands between 2007 and 2010 when a series of community infection cases emerged [28]. A high prevalence of spontaneous abortions in goats and sheep was identified as the primary source of infection in humans [29]. The outbreak affected a population with low previous Q fever seroprevalence [30]. Based on further studies, over 40,000 people were

thought to be infected with *C. burnetii*. Other than direct exposure to the infected farm animals, downwind contaminated aerosols from dairy farms also contributed to the spread [31-33]. In December, 2009, the Dutch government decided to systemically cull more than 50,000 gestating goats and ewes in an effort to control the disease [34]. This veterinary measure along with increased immunization of sheep and goats finally resulted in decreased number of reported human cases in 2010.

Bacteriology

Coxiella is a small 0.2 to 0.4 μm wide and 0.4 to 1 μm long coccobacillus. It has a cell wall similar to that of a Gram-negative bacterium but not stainable with the Gram technique. Instead, the Gimenez method is used to stain the bacterium in culture or in clinical isolates. Previous studies indicated that the estimated doubling time for *Coxiella* is ~12 h in *in vitro* conditions, in Vero cells [35]. Once internalized by eukaryotic host cells, this obligate intracellular pathogen resides in the phagocytic vacuole and modifies the vacuole to form a replicative niche for itself. The bacterium displays antigenic phase variation due to the mutational variation in its membrane lipopolysaccharide (LPS) [36]. As a result, the bacterium can either have a phase I phenotype identified by the presence of complete LPS on cell surface or it can display a phase II phenotype where the bacterium expresses a truncated LPS which lack the O-antigen side chains [37]. This phase variation is known to be linked to bacterial virulence [38]. Phase I bacteria are virulent and capable of replicating in immunocompetent hosts, whereas avirulent phase II bacteria are successfully cleared out by host immune response. Phase I to phase II transition is primarily associated with the deletion of gene clusters that produce O-antigen

of LPS. This shift in phase can occur during repeated bacterial passages in laboratory *in vitro* cultures [39, 40].

The biphasic developmental cycle of *Coxiella* involves two forms or variants observed in *Coxiella* based on their size that are clearly distinguishable by electron microscopy [41]. The large cell variant (LCV) replicates exponentially and is larger (>0.5 μm) than the stationary non-replicating small cell variant (SCV) (0.2-0.5 μm). SCVs are the extracellular form that are metabolically inactive and highly resistant to stress factors such as UV radiation, osmotic, mechanical, chemical, heat and desiccation stresses, which make them environmentally very stable so that they can survive in the environment for prolonged periods of time [42]. Probably the high number of peptidoglycan crosslinks in the membrane confer the stress-resistance property of SCVs [43]. LCVs on the other hand, are the metabolically active, intracellular form that is less resistant to the environmental stress [41]. The dispersed chromatin in LCV in contrast to the condensed chromatin of SCV is also a key difference between the two forms [43].

The SCVs are observed intracellularly in long term (21 to 28 days) culture of Vero cells. The developmental transition and viability of *Coxiella* were also seen in axenic acidified cysteine citrate medium 2 (ACCM2) [44]. This ability of transitioning developmental features contributes to the pathogenic efficiency of the bacteria.

Immunity, treatment, and vaccine

In its acute form, Q fever is self-limiting and readily resolves without antibiotic intervention. The recommended treatment of more life-threatening chronic disease is complex, as the only effective therapy requires a daily antibiotic combination of doxycycline, a protein synthesis inhibitor along with the alkalizing agent

hydroxychloroquine for a minimum of 18 months [45, 46]. This combinatorial treatment regimen was shown to be most effective in preventing relapses as well [46]. Administration of hydroxychloroquine circumvents resistance against doxycycline, but the recent reports of emergence of acquired doxycycline resistance against *Coxiella* emphasizes the need to understand and prevent the pathogenicity [47].

Discovery of *Coxiella* as the pathological agent behind Q fever simultaneously motivated researchers to develop a human vaccine against the bacterium. A live-attenuated vaccine was the first to be tested but production was eventually stopped as a result of long-term efficacy concerns [48]. Another chloroform-methanol residue extract vaccine developed in the US was not even tested in humans as it showed signs of reactions in animals [49]. Phase I LPS was known as a key determinant of protective immunity in humans, so when various attempts of developing a vaccine were being tested, inactivated whole cell phase I strain (Henzerling RSA 334) was a promising candidate for this purpose [50]. Further experiments proved that vaccine prepared from phase I bacteria was able to elicit better immune response in humans and animals than its phase II counterpart [51, 52]. Thus, a whole-cell formalin-inactivated Q fever vaccine (Q-Vax) was developed which showed a 98% efficacy, a lifelong immunity and was safe [50, 53]. However, currently Australia is the only country employing Q-Vax to prevent *Coxiella* outbreak because individuals exposed to *Coxiella* previously show adverse reactions to vaccination. Vaccination with whole-cell bacterium has been shown to cause severe local (at the inoculation site) and sometimes systemic reactions [54, 55]. In order to prevent such adverse reactions, a screening process has to be completed to test the seroprevalence, before clearing the individual to receive the vaccine. This creates a major

logistic hurdle in large-scale vaccination strategy. In addition to this, requirement of a highly contained BSL-3 laboratory facility for production of this whole - cell vaccine makes the vaccine expensive to produce and reproducibility is challenging. A subunit vaccine would probably be more efficient in controlling the disease outbreak.

Coxiella burnetii Type IVB Secretion system

The majority of intracellular bacterial pathogens possess a specialized secretion system which transports proteins across their replicative vacuoles, into the host cytosol. These bacterial effector proteins are required to interact with their environment and are used to modulate an array of host processes. Dot/Icm proteins of the type 4B secretion system (T4BSS) are essential for *Coxiella* virulence [56]. The *Coxiella* T4BSS Dot/Icm system injects about 130 effector proteins into the cytosol of infected host cells [57]. These proteins manipulate different host biochemical, metabolic, inflammatory and signaling pathways such as prevention of host cell death, interplay with macrophage inflammatory response and interaction with autophagosomes and are necessary for bacterial survival, replication, over all virulence; as well as in the development and maintenance of the *Coxiella*-containing vacuole (CCV) [58-60].

Studies involving *Legionella pneumophilia*, an evolutionarily related bacterium to *Coxiella* have indicated the presence of more than one secretion systems [59, 61-64]. Genomic analysis of *Coxiella* confirmed that secretion systems type I and II are also present in the bacteria, but there is not any study so far confirming their effector translocation across CCV membranes [65-67]. *Coxiella* encodes elements of a Sec-dependent secretion pathway, as well as a complete T4BSS, which translocate effector proteins across vacuolar and bacterial membranes and shape its replicative niche [67, 68].

Intracellular lifestyle of *Coxiella burnetii*

Although multiple intracellular resident phagocytic and non-phagocytic cells including neutrophils, fibroblasts, epithelial cells, endothelial cells, and interstitial/alveolar macrophages are present in the lung environment, alveolar macrophages remain the primary and preferential target of *Coxiella* infection[69, 70]. *Coxiella* enters the cell by a passive actin-dependent phagocytosis. Once internalized, instead of using mechanisms to escape endocytic pathways, *Coxiella* remains encased in its phagosome and eventually modifies it to develop a lysosome-like replicative niche [71]. This compartment, called the *Coxiella* containing vacuole (CCV) matures along the endocytic pathway acquiring various markers of the endolysosomal system (Figure 2) [72-75]. Interestingly, this initial part of the endocytic pathway is host canonical and does not require bacterial modulation, which is consistent with the observation that *Coxiella* Dot/Icm (defect in organelle trafficking/intracellular multiplication) system is not functional at this early stage of infection [60, 71, 76]. It is speculated that *Coxiella* has evolved this fine-tuned mechanism to regulate the Dot/Icm system effector secretion such that it can avoid host immune signaling during the early stage of infection process [71].

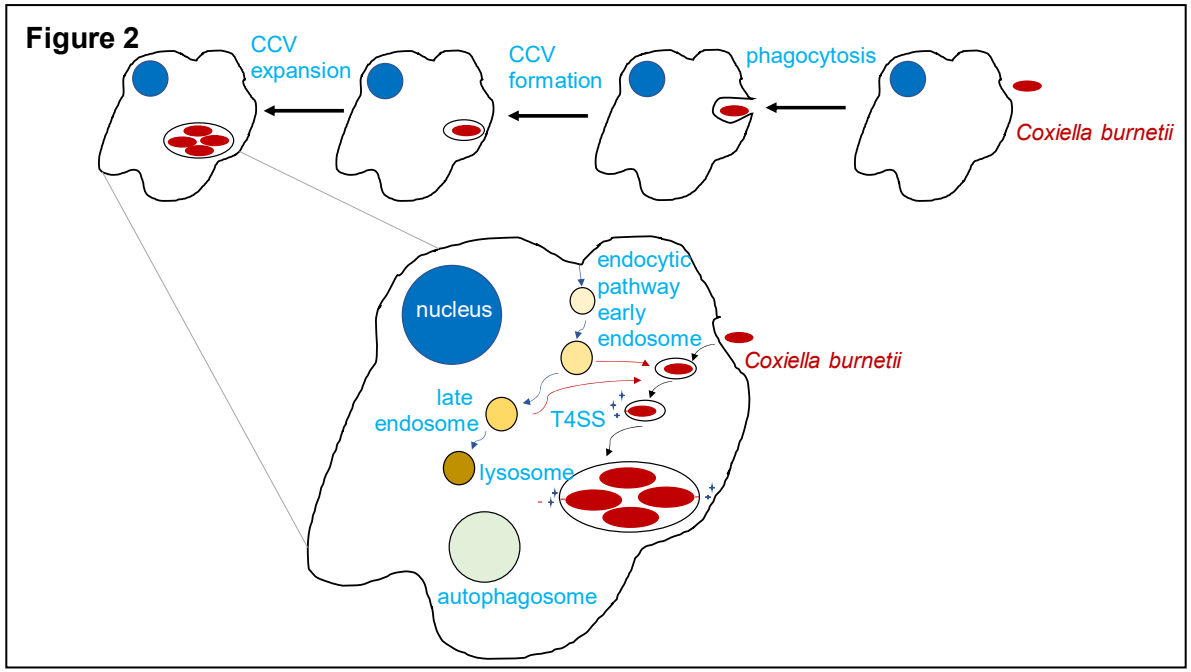

Figure 2: Intracellular lifestyle of *Coxiella*

Coxiella is phagocytosed primarily by macrophages, but non-phagocytic cells such as fibroblasts and epithelial cells can also take up *Coxiella.* Uptake of *Coxiella* is followed by maturation of the nascent tight-fitting phagocytic vacuole via its fusion with various host endocytic components. Bacteria grow in the expanding CCV over time and T4BSS effector proteins start to translocate across the CCV membrane, into the host cytoplasm.

During its maturation, the CCV progresses through the host endocytic pathway traffic and fuses with host late endosome and lysosomal vesicles, thus forming an expanded and large phagolysosomal compartment. Moreover, as a result of this, the CCV acquires different endosomal markers indicative of their respective maturation stages, along with progressive acidification of the vacuole [75].

Whereas intracellular pathogens such as *Toxoplasma gondii*, *Chlamydia trachomatis* and *Legionella* avoid the bactericidal endosomal maturation altogether, *Coxiella* survives in the phagolysosomal environment derived from fusion of CCV with different endocytic vesicles [75, 77]. Earlier biochemical studies have also proved that at a neutral pH of 7, *Coxiella* undergoes minimal metabolic activity, but acidification of the media stimulated glucose metabolism [78, 79]. Thus, acidification of nascent phagosome is required to activate bacterial metabolism and secretion system [58]. CCV acidification is required for successful effector delivery by *Coxiella* [79]. As early as 8 hours post infection, once the bacterium is in the acidified environment of a late endocytic organelle, the T4BSS system is activated and effector proteins start to translocate across the CCV membrane into the host cytosol [61, 80, 81].

However, *Coxiella* is unique in its active, T4BSS-dependent regulation of the CCV acidification process, as demonstrated by recent findings from our lab that not only is CCV less acidic than a mature lysosome, but *Coxiella* is so sensitive to more acidic environment, that further lowering of the pH of the CCV causes bacterial lysis [58, 82]. Furthermore, using a T4BSS mutant deficient in Dot/Icm function (Δ*dotA*), we were able to prove that pH of mature endosomes is manipulated by *Coxiella* and the endosomes from Δ*dotA Coxiella* infected are significantly more acidic than the endosomes from WT

Coxiella infected cells [58]. This was a surprising and groundbreaking finding because this observation suggested that there were reduced number of proteolytically active lysosomes available to fuse with CCV as a result of less acidic terminal endocytic vesicles present in infected cells than in the uninfected ones, as a mechanism to modulate CCV pH. Indeed, cathepsin B Magic Red assay to identify proteolytically active lysosomes revealed that WT *Coxiella*–infected cells had significantly reduced cathepsin B activity than the mock-infected controls [58]. This observation changed the long-standing notion that even though CCV is a lysosome-derived compartment, and the initial CCV acidity is necessary to drive *Coxiella* metabolism, it is not as acidic as a lysosome. We confirmed that *Coxiella* regulates CCV and endosomal pH in infected host cells and actively regulates CCV pH at a stable range of ~5.2 during a 6 day infection [58].

Starting from the initial observation that increasing acidity by decreasing CCV pH is detrimental for *Coxiella*, we established that in contrast to previous belief, lysosomes and terminal endocytic vesicles are less acidic in the infected cells, which results in formation of less acidic CCV from heterotypic fusion, and this process is regulated by *Coxiella* actively. Our findings were further reinforced by the observation that increasing lysosomal content of a cell by enhancing lysosomal biogenesis significantly inhibited *Coxiella* growth [58]. However, how *Coxiella* subverts host lysosomes is not fully understood.

Lysosomal biogenesis and Transcription factor EB (TFEB) in relation to *Coxiella*

Lysosomal genes primarily belong to a network called Coordinated Lysosomal Expression And Regulation (CLEAR) [83]. Promoter region analysis of 96 known lysosomal genes indicated that 68 of them have a palindromic 10 bp consensus region

[84]. This motif was located within 200 bp of transcription start sites (TSS) in humans suggesting their role in transcriptional activation of these genes and is known as the CLEAR motif. Transcription factor EB (TFEB) directly binds to this promoter site and thus, is known as master regulator of the CLEAR network. One or more in tandem CLEAR motif(s) have been identified in the promoter region of many lysosomal genes, including those that encode for lysosomal membrane proteins, hydrolases, accessory proteins, and biogenesis proteins. As depicted in figure 3,the majority of the genes in the CLEAR network are involved in lysosomal biogenesis and function (genes encoding subunits of vacuolar proton pump, responsible for creating and maintaining lysosomal acidic environment, lysosomal membrane proteins and autophagy proteins) as well as in synthesis of lysosomal hydrolase, transporter and accessory proteins [85]. Table #1 lists the genes that are TFEB direct targets with known lysosomal functions [85]. TFEB overexpression results in upregulation of 291 genes, most of which are related to lysosomal biogenesis and function further emphasizing the role of TFEB in lysosome homeostasis in the cell [86]. TFEB is a therapeutic target for lysosomal storage disorders, neurodegenerative diseases, and different types of cancer [87-90]. Interestingly, a recent study with two intracellular bacteria, *Mycobacterium tuberculosis* and *Burkholderia cenocepacia* have shown that TFEB induced lysosome biogenesis boosted the ability of macrophages to eliminate the pathogen, methicillin-resistant *Staphylococcus aureus* in a murine model [91].

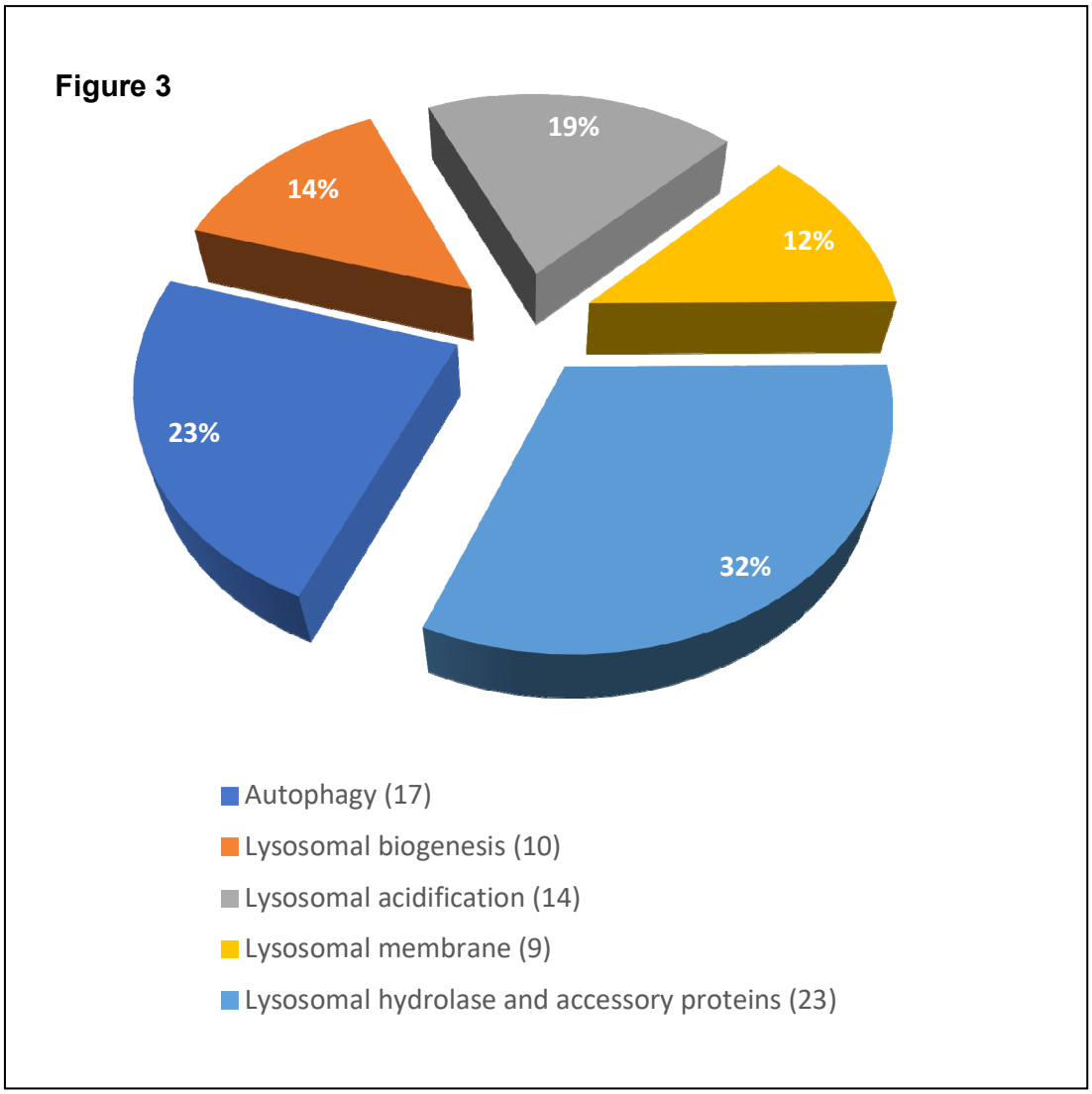

Figure 3: Lysosomal functional categories controlled by CLEAR network

Characterization of CLEAR network revealed gene categories associated with lysosomal function, number of TFEB targets indicated in parenthesis [85].

Table 1

Category	Gene name
Lysosomal hydrolases and accessory proteins	ASAH1, CTSA, CTSB, CTSD, CTSF, GAA, GALNS, GBA, GLA, GLB1, GNS, GUSB, HEXA, HEXB, IFI30, NAGLU, NEU1, PLBD2, PPT1, PSAP, SCPEP1, SGSH, TPP1
Lysosomal membrane	C1orf85, CD63, CLCN7, CLN3, CTNS, MCOLN1, SLC36A1, LAMP1, TMEM55B
Lysosomal acidification	ATP6AP1, ATP6V0A1, ATP6V0B, ATP6V0C, ATP6V0D1, ATP6V0D2, ATP6V0E1, ATP6V1A, ATP6V1B2, ATP6V1C1, ATP6V1D, ATP6V1E1, ATP6V1G1, ATP6V1H
Non-lysosomal proteins involved in lysosomal biogenesis	NAGPA, GNPTG, IGF2R, M6PR, BLOC1S1, BLOC1S3, HPS1, HPS3, HPS5, SUMF1
Autophagy	BECN1, GABARAP, HIF1A, NRBF2, PRKAG2, RAB7A, RRAGC, SQSTM1, STK4, UVRAG, VPS8, VPS11, VPS18,VPS26A, VPS33A, VPS35, WDR45

Table 1: TFEB direct target genes with known roles in lysosome function

List of known genes that are targeted by TFEB [85].

TFEB also regulates another important eukaryotic cellular clearance process, autophagy, by which damaged organelles, invading pathogens and defective cellular components including misfolded proteins are cleaned out of cells. The *Coxiella*-containing nascent tight-fitting phagosome interacts with autophagosomes and displays LC3 protein, a marker of autophagic vacuoles at a time point as early as 18 h post-infection [92]. Early stage interference of autophagy using inhibitors blocked CCV formation[92]. These findings showed that CCV formation is closely related with autophagy and *Coxiella* utilizes this host machinery to thrive. Autophagy related genes are transcriptionally regulated by TFEB and as a result TFEB activation results in a remarkable increase in autophagic flux [85, 93]. However, recent work from our lab has shown that *Coxiella* reduces lysosomes in infected cells in a manner independent of autophagy [94].

This observation further supports our hypothesis that *Coxiella* blocks CCV acidification by impeding lysosomal biogenesis, which is mediated by inhibition of TFEB activation. TFEB mediated promoter binding and elevated gene expression is dependent on its cytosol-to-nucleus translocation. TFEB activation and the resulting translocation are largely dependent on the phosphorylation status of TFEB. Phosphorylated TFEB is sequestered into the cytosol, thus the transcriptional induction of its target genes is inhibited. Conversely, when TFEB is dephosphorylated, it rapidly translocates to the nucleus, where it binds to the promoter regions of its target genes, thus initiating their transcription. There are several kinase regulators such as GSK3B, AKT, mechanistic target of rapamycin (mTOR) and ERK2, as well as phosphatases, such as calcineurin, that modulate TFEB phosphorylation by acting on the key TFEB amino acid residues. mTOR

is one of the most important kinases regulating TFEB phosphorylation, since both ERK and GSK3 kinases are not able to phosphorylate TFEB in the absence of mTOR activity [95]. Thus, mTOR mediated TFEB phosphorylation is a key event that determines subcellular TFEB distribution (Figure 4) [95-100].

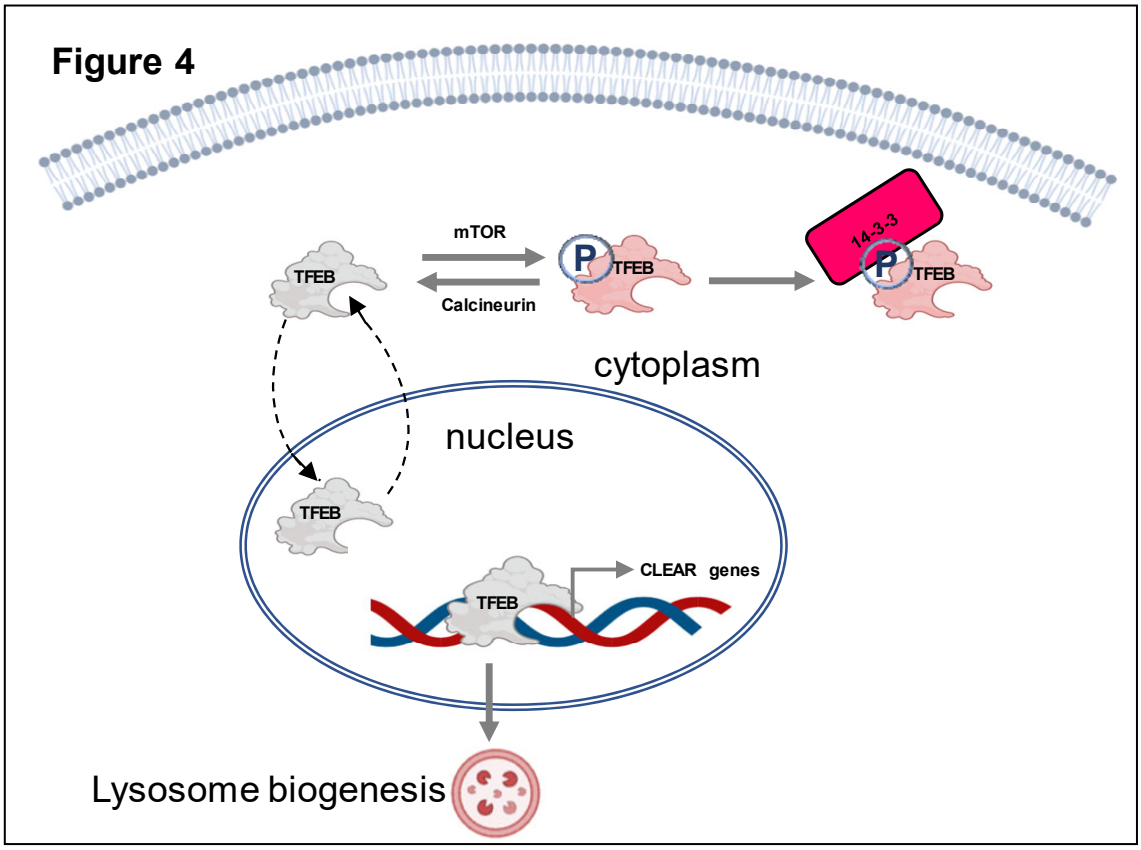

Figure 4: Schematic diagram describing mechanisms by which TFEB is activated and lysosome biogenesis is initiated

A substantial fraction of endogenous cytosolic TFEB is phosphorylated under basal conditions, thus inactive. Dephosphorylated TFEB translocates to nucleus, where it actively functions as the master regulator of the CLEAR network by inducing the transcription of lysosomal genes after binding to their promoter sequence. Retention of TFEB in the cytosol is determined by its mTOR-mediated phosphorylation. Phospho-TFEB complexes with 14-3-3, a chaperone protein, masks nuclear localization signal and thereby barring TFEB nuclear entry. Dephosphorylation of TFEB by calcineurin, a protein phosphatase, results in dissociation of TFEB from 14-3-3, and TFEB readily translocates to the nucleus, resulting in activation of the CLEAR network.

Given our finding that *Coxiella* actively regulates the CCV pH by manipulating the host cell in a way that results in reduced number of mature endosome/lysosome available for fusion with CCV and the role of TFEB in lysosome biogenesis, we rationalized that increased lysosome biogenesis by TFEB overexpression might have a detrimental effect in *Coxiella* viability. Indeed, published reports from our lab showed that CCVs in TFEB overexpressing Hela cells were significantly smaller in size and they never expanded over a 6 days experimental time period. Moreover, *Coxiella* growth was found to be significantly reduced in these TFEB overexpressing cells. pH measurement assay revealed that the CCVs in TFEB overexpressing cells were more acidic than the ones in parental cells. Taken together, these findings suggested that increased lysosome biogenesis as a result of TFEB overexpression resulted in increased acidification of CCV, leading to inhibition of intracellular *Coxiella* growth.

Research goals

CCVs, even though they are derived from host endocytic pathways, are distinctly different from the mature lysosomes of the host cell. In two different seminal studies from our lab, we showed that acidification of CCV by cholesterol accumulation in CCV membrane led to bacterial lysis. Further, we determined that the CCV and mature endocytic vesicles of *Coxiella*-infected cells are remarkably less acidic than the lysosomes of uninfected cells and biogenesis of host lysosomes by TFEB overexpression is detrimental for *Coxiella* growth [58, 82]. Based on these observations our next logical step was to test whether decreased lysosome biogenesis by complete TFEB knockout had any growth effect on *Coxiella*, followed by understanding the mechanism more comprehensively by which *Coxiella* manipulates the host endocytic pathway in its favor. Understanding this mechanism could be useful in discovering more efficient therapeutics for Q fever treatment in long term. With this aim in mind, we wanted to examine whether the lysosome biogenesis pathway is altered in *Coxiella* infected cells and how the bacterium is modulating it.

As described before, TFEB is the master regulator of lysosome biogenesis. Regulation of TFEB involves a dynamic process related to its subcellular localization. The shuttling of TFEB between cytoplasm and the nucleus is regulated by mTOR. Based on our findings, we hypothesized that *Coxiella* blocks the activation of TFEB to prevent lysosomal maturation, so that it can maintain a pH just ideal for its growth.

In order to test this hypothesis and elucidate the mechanism, we took the following approaches. Firstly, we tested whether *Coxiella* growth is promoted in TFEB KO cells which have decreased expression of lysosomes. Secondly, we wanted to see

whether *Coxiella* infection inhibits TFEB nuclear translocation in the infected cells compared to the uninfected ones. Conducting these studies would allow us to understand the finely tuned mechanism by which *Coxiella* alters the host endocytic pathways in its favor.

CHAPTER TWO: MATERIALS AND METHODS

Bacterial strains and mammalian cells

mCherry-expressing *C. burnetii* NMII was purified from Vero cells (African green monkey kidney epithelial cells [ATCC CCL-81; American Type Culture Collection, Manassas, VA]) and stored as previously described [82]. The multiplicity of infection (MOI) of the bacterial stock was optimized for each cell type and culture vessel used to ~1 internalized bacterium per cell at 37°C and 5% CO2. Human cervical epithelial cells (HeLa, ATCC CCL-2) were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (Corning, New York, NY, USA) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, USA) and 2 mM L-alanyl-L-glutamine (glutagro; Cat. 25-015-CI, Corning, New York, NY) at 37°C and 5% CO2. The wild type (parental) and TFEB knockout HeLa cells (generously provided by Richard J. Youle) were maintained in DMEM (Dulbecco's Modified Eagle Medium; Corning) containing 10% FBS at 37°C and 5% CO2 [101].

Lysosome-associated membrane protein 1 (LAMP1) immunostaining

Wild type (parental) and TFEB KO HeLa cells were seeded on coverslips in 24-well plates (5 X 10^4 cells per well, three wells for each cell type). The next day cells were fixed in 2.5% paraformaldehyde (Cat. 15710, Electron Microscopy Sciences; Hatfield, PA, USA) for 15 min and blocked/permeabilized for 20 min in 1% bovine serum albumin (BSA) and 0.1% saponin in PBS. Cells were then incubated with rabbit anti-LAMP1 (1:1000; Cat. ab24170 Abcam, Cambridge, MA) for 1h followed by Alexa fluor 568 secondary antibody (1:1000; Life Technologies) for 1 h. Following washing with PBS, coverslips were mounted using ProLong Gold with 4', 6'-diamidino-2 phenylindole

(DAPI) (Life Technologies) and visualized on the fluorescent microscope using oil immersion objective as described previously [58]. Images were captured and processed identically and the fluorescent intensity of LAMP1 was measured (ImageJ). At least 30 cells were measured per condition for each of three independent experiments.

Microscopy

Immunofluorescent images were obtained using a Nikon TiE fluorescent microscope (60 X oil immersion objective) and at least 30 CCVs and cells were visualized for each condition during three biological replicate experiments. All images were analyzed using the FIJI ImageJ software.

TFEB immunoblotting

Parental and TFEB KO HeLa cells were seeded in a 24-well plates ($2.5X10^4$ cells/well). The next day cells were lysed with 2% sodium dodecyl sulphate (SDS) in tris-buffered saline (TBS), and analyzed by immunoblotting to confirm TFEB KO. Protein lysates were resolved in 10% SDS-PAGE and transferred to PVDF membrane (Cat. IPFL00010, Millipore, Burlington, MA). The membrane was then probed using rabbit anti-TFEB antibody (1:1000; Cat. 37785, Cell Signaling Technologies, Danvers, MA) in 1% BSA in PBS, where GAPDH was used as loading control. After washing, the blot was incubated with horseradish peroxidase-conjugated anti-rabbit (1:1000; Cat. 31460, Thermo Fisher) or anti-mouse (1:1000; Cat. 31430, Thermo Fisher) secondary antibody in 4% non-fat milk in TBS-T (TBS containing 0.05% tween-20), and developed using enhanced chemiluminsence (ECL) reagent (SuperSignal West Pico PLUS; Cat. 34580, Thermo Scientific, Rockford, IL).

Subcellular fractionation

HeLa cells harvested 3 days post-infection with WT *C. burnetii* were subjected to a detergent based cellular fractionation using cytoplasm and nucleus isolation buffers provided with the cell fractionation kit (Cat# 9038, Cell Signaling technology, Danvers, MA). Briefly, cells were lysed to collect whole cell lysate, cytoplasmic fraction and nuclear fraction. After the fractions were collected, they were probed for detection of TFEB by resolving the samples on SDS gel in an immunoblotting assay. Purity of the fractions were also checked by probing them with cytosolic or nuclear markers, such as GAPDH and histone, respectively.

Colony forming unit (CFU) assay

Parental and TFEBKO HeLa cells were plated in 6-well plate (2 X 10^5 cells per well) and allowed to adhere overnight. The cells were infected with wild type *C. burnetii* in 0.5 mL DMEM for 2 h, washed extensively with PBS, and 2 mL of fresh 10% DMEM was added to each well. Infected cells were replated into a 24-well plate (2.5 X 10^4 cells/well for day 3, and 5 X 10^3 cells/well for day 6). To determine day 0, a fraction of infected cells was collected and lysed in sterile water for 5 min. The released bacteria were diluted 1:5 in ACCM-D and plated in 5-fold serial dilutions onto 0.25% ACCM-D agarose plates [58]. For the subsequent time points, the cells were lysed in sterile water for 5 min and the released bacteria were diluted 1:5 in ACCM-D and spotted in 10-fold serial dilutions onto 0.25% ACCM-D agarose plates. The plates were incubated for 7 to 9 days at 37°C in 2.5% O2 and 5% CO2, and the number of colonies counted to measure bacterial viability. Number of day 3 and day 6 colonies were normalized over that of day

0 CFU. Each of the three experiments was performed in biological duplicate, and the bacteria were spotted in triplicate.

Quantification of CCV size

Parental and TFEBKO HeLa cells were plated in a six well plate (2 X 10^5 cells/well) and allowed to adhere overnight. Cells were infected with wild type mCherry-WT *C. burnetii* for 2 h, washed extensively with PBS, and scraped into 2 mL of 10% DMEM. Infected cells were replated onto coverslips in a 24 well plate (2.5 X 10^4 cells/well for day 2, and 5 X 10^3 cells/well for day 6). At the indicated time points, cells were fixed with 2.5% PFA for 15 min, washed in PBS, and blocked/permeabilized in 1% BSA and 0.1% saponin in PBS for 20 min. Coverslips were stained with mouse anti-CD63 (1:1000; Cat. 556019; BD Biosciences, San Jose, CA) for 2 h, followed by Alexa Fluor 647 secondary antibody (1:1000; Invitrogen) for 1 h [58]. Following washing with PBS, coverslips were mounted with ProLong Gold with DAPI and visualized on a Nikon TiE microscope (60X oil immersion objective). Images were captured and processed identically, and the CCV area was measured using ImageJ software. At least 30 CCVs were measured per condition for each of three independent experiments.

Statistical analysis

Statistical analyses were performed using the Prism software using unpaired student t-test, or ordinary one-way ANOVA (with Tukey's correction) as appropriate in GraphPad (GraphPad Software, Inc., La Jolla, CA).

CHAPTER THREE: RESULTS

Validation of decreased lysosome biogenesis in TFEB KO cells

Recent published work from our lab showed that our TFEB overexpressing TFEB-GFP cells have increased proteolytically active lysosomes as was evident from cathepsin B activity compared to the parental cells [58]. This observation agrees with a previously reported study where TFEB overexpression resulted in increased lysosomal biogenesis as indicated by LAMP1 immunostaining in a stably transfected TFEB overexpressing HeLa cell system [84]. Infection of these cells with *Coxiella* revealed that increased lysosomes in host cells is detrimental for its growth [58]. Part of the long-term goal of the current study is to determine if *Coxiella* can block TFEB activation leading to inhibition of lysosomal biogenesis. To this end, examining the effect of complete loss of endogenous TFEB expression on *Coxiella* growth would provide us some insight on the bacterial viability in decreased lysosome condition. An approach to test that would be to employ TFEB KO HeLa cells generated by CRISPR/Cas9-mediated gene KO system and infect them with WT *Coxiella* [101]. Before conducting *Coxiella* viability experiments with TFEB KO cells, we wanted to validate these stable cells by testing their lysosomal content. We used LAMP-1 immunostaining to quantify lysosomes and compare the expression of LAMP-1 intensity. Parental HeLa cells were used as control. After identically acquiring the images of the cells, the LAMP-1 intensity was measured and quantified in ImageJ software. As expected, we observed that there was significantly less LAMP-1 expression in the TFEB KO cells (Figure 5 A and B).

We also validated the TFEB KO cells by performing immunoblotting for TFEB expression. As expected, lysate from TFEB KO cells showed no TFEB expression,

confirming complete TFEB has been disrupted in these cells (Figure 5 C). These two experiments proved that our TFEB KO cell system to be tested further with *Coxiella* infection model indeed has decreased lysosomes as a result of TFEB knockout.

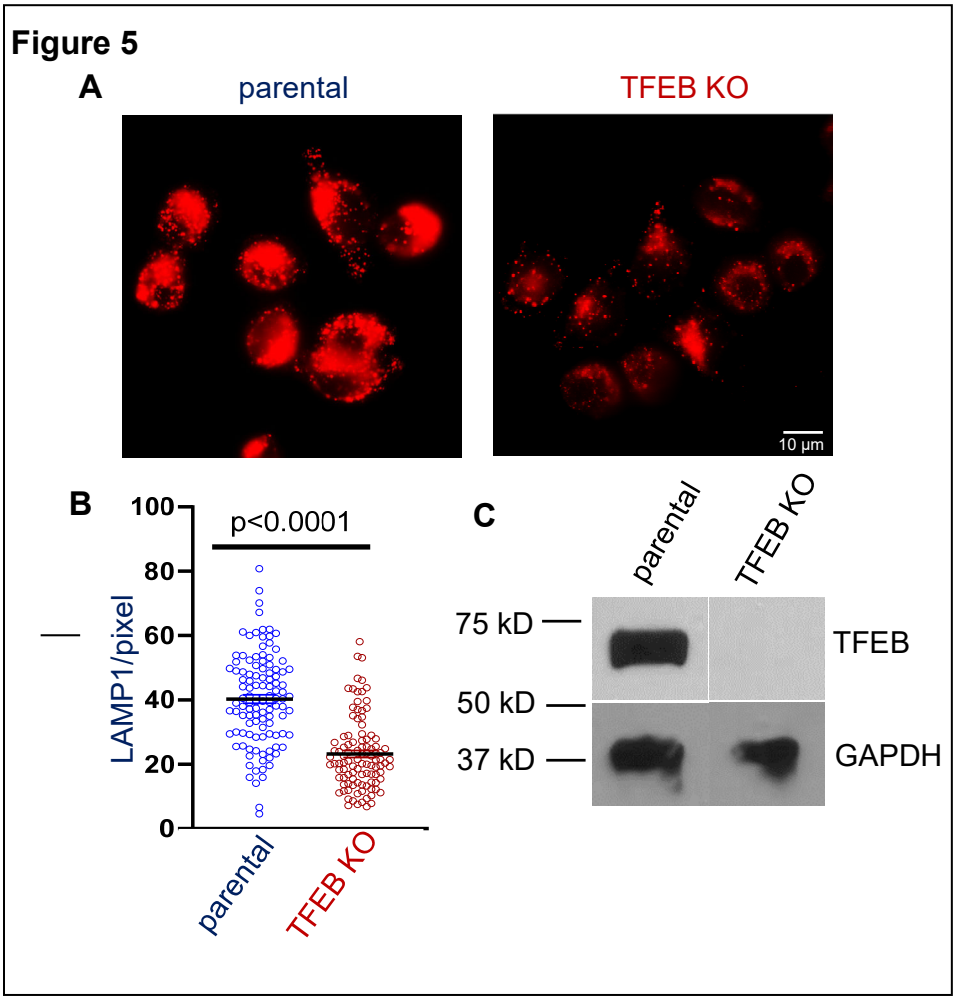

Figure 5: TFEB knockout results in decreased lysosomal biogenesis in HeLa cells

A. Representative images of LAMP1(lysosome marker) immunofluorescent staining in parental and TFEB KO cells. B. Quantitation of LAMP1 intensity in the parental and TFEB KO cells (each circle represents an individual cell) revealed decreased lysosomal biogenesis in the TFEB KO cells compared to the parental cells. Data shown as mean ± SEM of at least 30 cells per condition in each of three independent experiments as analyzed by student's t-test. C. Immunoblotting of whole cell lysate confirmed that there was no TFEB expression in the TFEB KO cells. GAPDH was used as loading control.

TFEB knockout results in bigger CCV size

Previous study from our lab using WT *Coxiella*-infected TFEB overexpressing and parental cells showed that TFEB-induced lysosome biogenesis inhibited *Coxiella* growth [58]. To test how ablation of TFEB expression affects *Coxiella* growth, parental and TFEB KO cells were infected with mCherry-expressing *Coxiella.* Immunostaining with CD63, a CCV marker was performed. Cells were counter-stained with DAPI. Immunofluorescent images were analyzed by microscopy 3 days and 6 days post-infection (dpi) and the images were analyzed on ImageJ software. CCV size was significantly bigger in TFEB KO cells compared to the parental (Figure 6). Bigger CCV size in TFEB KO cells indicated that decreased lysosomal biogenesis in host cells favors *Coxiella* growth.

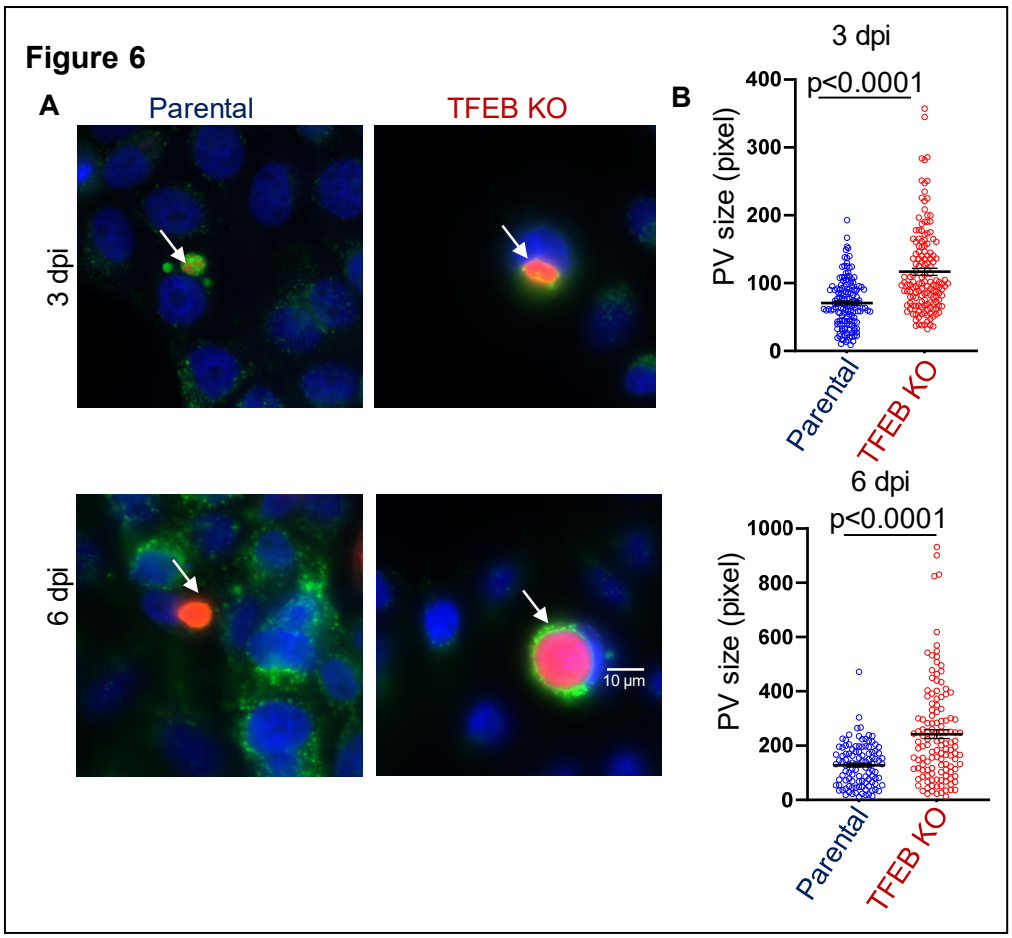

Figure 6: TFEB knockout results in bigger CCV size

A. Representative images of immunofluorescence staining of WT mCherry expressing *C. burnetii*-infected parental and TFEB KO HeLa cells at 3 days and 6 days post-infection (dpi). Fixed cells were stained for CD63, a CCV marker. Arrows point to individual CCVs. Qualitatively, TFEB KO cells contained bigger CCVs compared to parental cells at both time points. B. Quantitation of CCV size in parental and TFEB KO cells revealed significantly bigger CCVs in TFEB KO cells compared to parental cells at both time points. Each circle represents an individual CCV. Data shown as mean ± SEM of at least 35 CCVs per condition in each of three independent experiments as analyzed by unpaired Student's t-test.

TFEB knockout promotes *Coxiella* growth

Given that the CCV size is considerably bigger in TFEB KO cells infected by WT *Coxiella*, we sought to test whether this observation reflects in *Coxiella* growth as well. Quantitative agarose-based colony forming unit (CFU) assay showed that compared to parental cells, *Coxiella* growth was significantly higher at both 3 and 6 dpi timepoints in the TFEB KO cells (Figure 7). Data depicted in figures 6 and 7 suggested that decreased lysosomal biogenesis in TFEB KO HeLa cells promote *Coxiella* intracellular growth accompanied by larger CCV formation. These data are also in agreement with previous findings from our lab where we had observed TFEB-induced lysosome biogenesis inhibited *Coxiella* growth.

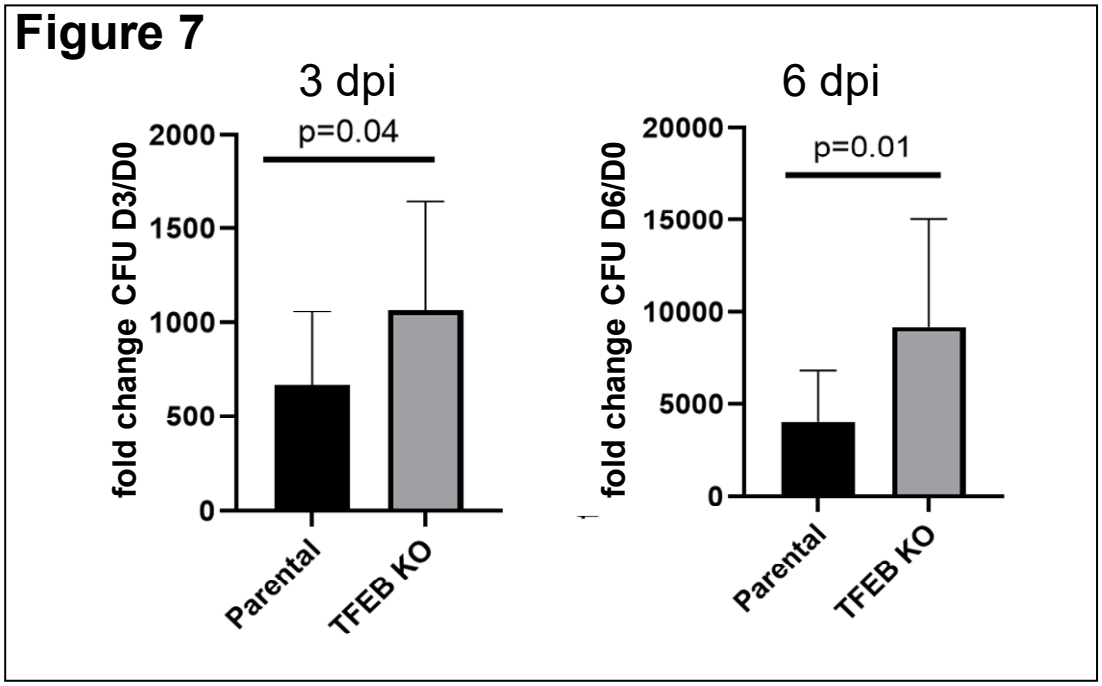

Figure 7: TFEB knockout promotes bacterial growth

A and B. Quantitative CFU assay revealed a significant increase in bacterial growth in TFEB KO cells compared to parental cells. *C. burnetii* growth is plotted as a fold change of CFU over day 0 well at each time point. Data shown as mean ± SEM from six independent experiments as analyzed by Student's t-test.

Coxiella infection inhibits torin-induced TFEB nuclear translocation in transiently transfected cells (induced TFEB expression)

Our findings that *Coxiella* growth is enhanced in TFEB KO cells, suggested that *Coxiella* manipulates the host cells in a way that lysosome biogenesis is inhibited upon infection. TFEB is the master regulator of lysosomal genes and its functional activation is regulated by phosphorylation and subsequent subcellular localization. TFEB phosphorylation is mediated by mTOR. Thus, we hypothesized that in WT *Coxiella* infected cells TFEB activation is inhibited.

To test this hypothesis, we transfected HeLa cells with a TFEB-GFP plasmid and infected these cells with WT *Coxiella*. At three days post infection, cells were treated with torin for two hours in a dose–dependent manner (data not shown). Torin is a kinase inhibitor drug and torin-mediated nuclear translocation of TFEB is a well-studied model in mTOR research area. There are multiple studies exploiting the mTOR inhibitory property of torin [101-103]. Essentially, torin inhibits mTOR by targeting its catalytic activity, thus causing a significant increase in nuclear translocation of TFEB and activating it. Microscopically, the reduction in diffused cytoplasmic pool of TFEB signal can be observed after torin treatment [104]. Torin-treated uninfected cells showing massive TFEB nuclear localization served as a positive control in this experiment. Controls also included i) DMSO (vehicle) treated cells, ii) untreated and uninfected but transfected cells, and finally iii) non-transfected and untreated cells (negative control). Cells colocalized with TFEB-GFP (transfected) and DAPI stain were deemed as positive for TFEB nuclear translocation. % TFEB translocated to nucleus was quantified by measuring the intensity of green TFEB signal in cytoplasm and nucleus using ImageJ

software. Images were analyzed using ImageJ software. WT *Coxiella* infected cells have less TFEB translocated to the nucleus than the uninfected cells (Figure 8).

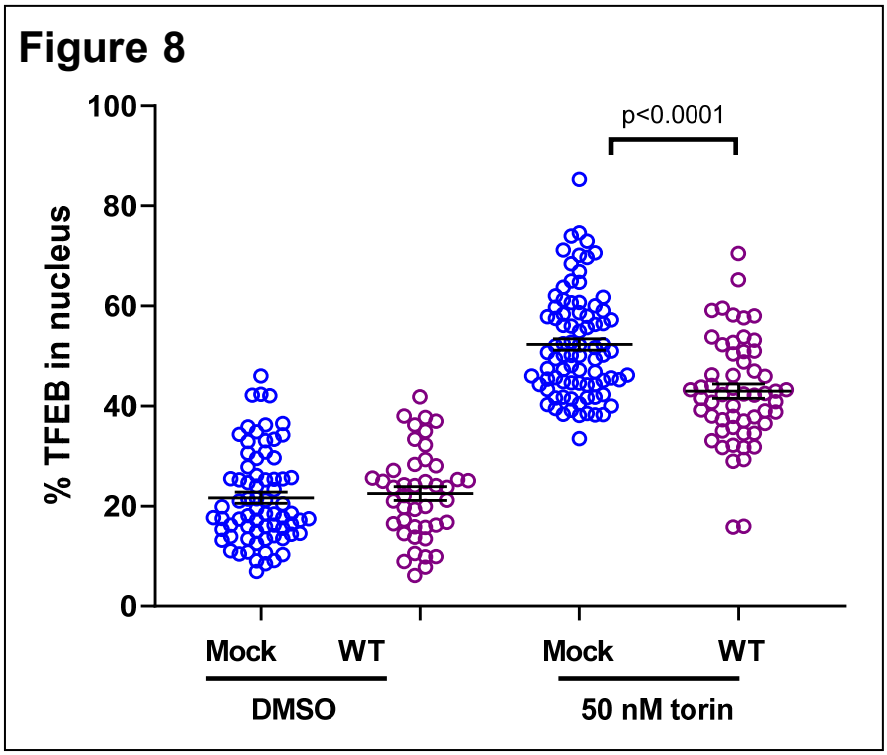

Figure 8: *Coxiella* infection inhibits torin-induced TFEB nuclear translocation (activation) in transiently transfected HeLa cells (induced TFEB expression)

HeLa cells were transfected with TFEB-GFP plasmid and 24 hours post transfection, WT mCherry expressing *Coxiella*-infected HeLa cells were treated with DMSO or torin 3 days post-infection. Cells were then fixed and stained with DAPI before imaging. As seen from the quantitation, there was decreased torin-induced TFEB translocation to nucleus in the infected cells compared to the uninfected cells. In other words, there was less activated TFEB in the infected cells than the uninfected cells. At least 30 cells/condition were imaged. Data shown as mean ± SEM from four independent experiments as analyzed by Student's t-test.

Coxiella infection inhibits torin-induced TFEB nuclear translocation in cells (endogenous expression)

In order to determine whether the inhibited nuclear translocation of TFEB in transiently induced HeLa cells upon WT *Coxiella* infection is also observed in cells endogenously, we tested the presence of TFEB protein in cytosolic and nuclear fractions of the uninfected and WT *Coxiella*-infected cells through immunoblotting. We used an optimum dose of 50 nM torin as we did for the previous experiment. We used torin and DMSO controls as described previously. In this cell fractionation-based assay, immunoblotting of nuclear and cytosolic fractions for TFEB was performed. We used GAPDH and histone as cytosolic and nuclear markers respectively, to test the purity of the fractions. As indicated in figure 9, there was a prominent decrease in the TFEB translocation in the nuclear fraction of infected cells. This experiment further confirmed that *Coxiella* caused an inhibition of host cell TFEB translocation to the nucleus, which may result in decreased transcription of CLEAR genes leading to less lysosomal biogenesis in the infected cells.

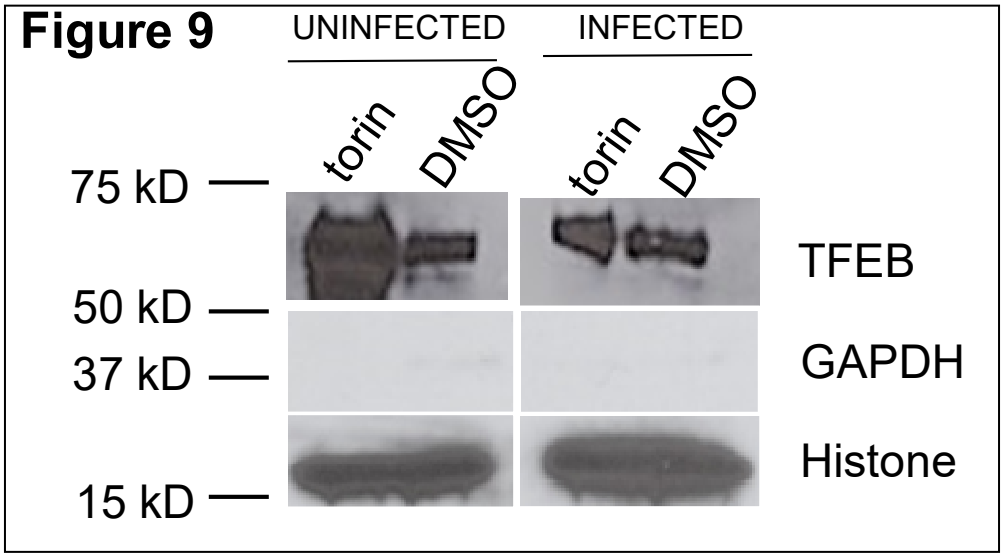

Figure 9: *Coxiella* infection inhibits torin-induced TFEB nuclear translocation (activation) in HeLa cells (endogenous expression)

Immunoblot for total endogenous TFEB revealed that WT *Coxiella* infection inhibits nuclear TFEB translocation in HeLa cells treated with torin 3 days post infection. Compared to the uninfected cells, the infected cells showed less TFEB translocated to nucleus suggesting that *Coxiella* infection blocks torin-induced TFEB activation.

CHAPTER FOUR: DISCUSSION

Coxiella is a pathogenic intracellular bacterium which was long believed to be unique because it was thought to be residing and replicating in the degrative acidic environment of lysosomes. Unlike other intracellular pathogens, *Coxiella* depends on the host cell to be delivered to a lysosomal environment. Once the bacterium senses the acidic environment of an endocytic vacuole, its Dot/Icm secretion system and metabolism are activated, followed by initiation of effector secretion. The effector proteins play important roles in *Coxiella* pathogenesis and replication. Five effector proteins, referred to as *Coxiella* vacuolar proteins (Cvp A to E), for example, are localized at CCV and mutations in *cvp* genes affects CCV biogenesis and *Coxiella* replication [105]. Unlike other pathogens that are internalized in the cell by the host endocytic pathway and reside in multiple, small tight-fitting vacuoles, CCVs are highly fusogenic, and mature CCVs are often large enough to occupy the entire cell cytosol, pushing the cell organelles to a side. Homotypic fusion of CCVs are mediated by the host component syntaxin-17 and the bacterial component CvpB/Cig2. Syntaxin - 17 is an autophagy -related SNARE protein and this host-bacterial interaction is an example of how an effector protein manipulates and uses host system for its own benefit [106, 107]. Components of the host autophagic pathways are also involved in membrane repair caused by damage in CCV [108]. Moreover, induction of autophagy has shown to be beneficial for generation and maturation of CCV [109]. Thus, autophagy pathway is closely modulated by and linked to CCV development.

In a recent study from our lab, we were able to prove that even though CCV is a niche derived from the host endocytic pathway, and its maturation is closely linked with

the host endolysosomal system, it is not as acidic as a mature endosome. In fact, while the initial acidification of CCV is required by the bacterium to successfully reside and replicate in the host cell, further acidification is detrimental for its growth [60]. The observation that inducing CCV acidification to pH ~4.8 led to bacterial death led us to investigate the endosomal maturation in *Coxiella* infected cells. We found that CCV pH (~5.2) is significantly less acidic than the lysosomes of uninfected host cells (~4.8). Close examination and comparison between pH of uninfected and *Coxiella* infected lysosomes suggested that the pH of endosome in uninfected cells was 4.9, compared to the pH ~5.8 in infected cells. Not only this, increased lysosomal biogenesis led to decreased bacterial growth accompanied by smaller CCVs [58]. The current study reported in the scope of the thesis work is based on this seminal finding. We wanted to understand the mechanism by which *Coxiella* maintains a pH just right for its sustenance by reducing the availability of mature lysosomes in the infected cells.

Autophagy and lysosomal biogenesis are two crucial parts of the eukaryotic endolysosomal system. However, we found out that the reduction of lysosomes in infected cells is independent of autophagy and blocking autophagy did not affect lysosomal content [58]. So, lysosome biogenesis via regulation of TFEB was an important component that we wanted to investigate further. Experiments involving overexpression of TFEB and resulting increased lysosome biogenesis caused remarkably decreased viability of *Coxiella* [58], whereas complete TFEB ablation (Figures 5 and 6) or partial siRNA mediated TFEB silencing (unpublished data)resulted in increased bacterial growth. We observed similar trends in the CCV size - smaller CCVs where lysosome biogenesis was increased, larger CCVs where lysosome biogenesis was

compromised. These observations proved our hypothesis that *Coxiella* manipulates lysosome biogenesis possibly by modulating the TFEB mediated regulation of the CLEAR network.

Under basal conditions, a substantial fraction of total TFEB is phosphorylated and cytosolic, and thus inactive. Nuclear TFEB, on the other hand, is dephosphorylated and actively functions as the master regulator of the CLEAR network by inducing the transcription of lysosomal genes after binding to their promoter sequence. Retention of TFEB in the cytosol is determined by its mTOR-mediated phosphorylation status. Phospho-TFEB complexes with a chaperone protein 14-3-3, thus masking a nuclear localization signal (NLS), thereby precluding TFEB nuclear entry. Dephosphorylation of TFEB by calcineurin results in dissociation of TFEB from 14-3-3, which readily promotes its translocation to the nucleus, resulting in activation of the CLEAR network [110].

Several studies have shown that phosphorylation at specific serine residues (S211, S142 and S138) are specifically crucial for TFEB subcellular localization, and activation [93, 111, 112]. From our preliminary data using an antibody directed to the detection of total endogenous TFEB protein, we observed that there was decreased TFEB in the nuclear fraction of WT *Coxiella* infected HeLa cells 3 days post-infection, compared to their uninfected counterpart (Figures 8 and 9). Torin, which inhibits mTOR and causes a massive TFEB nuclear translocation was used a positive control, along with DMSO negative control. Cells were harvested 2 hours post-torin treatment. This interesting observation led us to conclude that even in a torin-induced condition, where TFEB is expected to be dephosphorylated and massively translocate to nucleus, *Coxiella* was able

to block the TFEB translocation to nucleus, thus blocking lysosome biogenesis (Figure 10). Future experiments will be focused on determining the phosphorylated and dephosphorylated TFEB ratio in the nucleus and cytoplasm of *Coxiella* infected cells and identifying the serine residues differentially phosphorylated in these cells. These data will provide further insight on the mechanism of fine-tuned regulation of lysosome biogenesis in *Coxiella* infected cells.

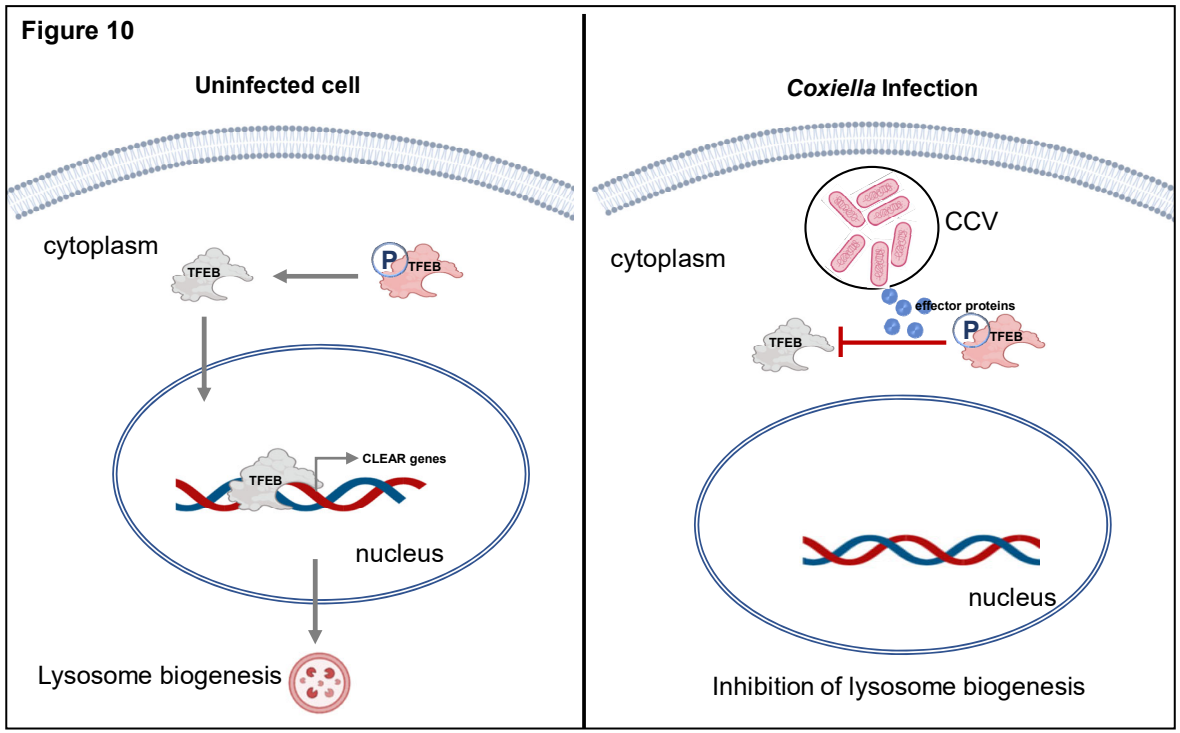

Figure 10: Summary of results

Coxiella is sensitive to acidic lysosomal environment and actively regulates CCV pH to maintain its growth and pathogenesis. While previous work from our lab has shown that *Coxiella* does this by inhibiting endosomal maturation, thus decreasing available acidic endosome/lysosome that can fuse with the CCV, this present work indicates that *Coxiella* might also manipulate lysosome biogenesis in infected host cells by blocking TFEB nuclear translocation.

For a more detailed and comprehensive understanding of this mechanism, phospho-specific antibodies should be used, which are able to recognize the phosphorylation state of specific amino acid residues. Phospho-specific antibodies for TFEB S142, TFEB S211 and TFEB S138 will be used in immunoblotting experiments. Understanding the mechanism behind TFEB inhibition upon *Coxiella* infection will be crucial in ways beyond *Coxiella* pathogenesis, such as in neurodegenerative disorders and cancer, where TFEB is induced or inhibited, respectively.

Previous studies have proved that translocation of effector proteins by *Coxiella* into the host cytosol are essential for a successful infection. About 354 candidate *Coxiella* effectors have been identified, 130 of which are secreted in a Dot/Icm dependent manner [113]. Previous data from our lab indicated that *Coxiella* effector proteins that manipulate host endosomal maturation are T4BSS dependent. So as part of the overall research goal, in the second part, we want to focus on T4BSS and work on identifying effector proteins targeting TFEB [58]. So, we will identify the protein using an unbiased screening approach. We expect to be able to identify effector protein belonging to the T4BSS, along with potential proteins belonging to other secretory pathway or system. Collectively, observations and findings from this study accompanied by the future scope of work described here will highlight the TFEB mediated lysosome biogenesis modulation in *Coxiella*-infected cells, which can be potentially useful beyond understanding *Coxiella* pathogenesis, such as in neurodegenerative disorders and cancer, where TFEB is induced or inhibited, respectively.

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CURRICULUM VITAE

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Education

- 2021: Master of Science in Microbiology and Immunology

  Indiana University-Purdue University Indianapolis, Indiana
- 2006: Master of Science in Biotechnology

  The University of Burdwan, India
- 2004: Bachelor of Science in Zoology

  The University of Burdwan, India

Research Experience

- 2018-2020: Graduate research student in Dr. Stacey Gilk's laboratory in Microbiology and Immunology department, Indiana University School of Medicine, Indiana
- 2015-2018: Research Assistant in Dr. Chandan K. Sen's laboratory in Department of Surgery at The Ohio State University Wexner Medical Center, College of Medicine.
- 2012-2015: Research Aide in Dr. Sashwati Roy's laboratory in Department of Surgery at The Ohio State University Wexner Medical Center, College of Medicine.
- 2011-2012: Research Volunteer in the laboratory of Prof. Sashwati Roy at the Department of Surgery, Ohio State University.
- 2009-2011: Clinical Research Coordinator on "A Double Blinded, Randomized, Four Arm, Dose Ranging Phase IIb Study to Assess the Efficacy, Safety and

Tolerability of Crofelemer in the treatment of acute infectious diarrhea" at the Institute of Post Graduate Medical Education & Research, Kolkata.

Awards and Honors

- 2017: Third place in Research Poster Competition at Dorothy M. Davis Heart & Lung Research Day, held at The Ohio State University, Columbus, Ohio.
- 2016: Selected for Research Critique at Fall SAWC, held at Las Vegas, Nevada.
- 2013: Excellence in Translational Regenerative Science Award at 23rd annual meeting of Wound Healing Society, held at Denver, Colorado

Patents

- 2020: Energy generation from fabric electrochemistry. WO EP US AU CA US20200006783A1
- 2019: Antimicrobial wound care dressing. WO EP US US20190247234A1

Invention disclosure

- 2017: Power Harvesting from Fabric Electrochemistry. OSU – ref P2017-121-01
- 2015: A Non-Contact Method for Accelerating Wound Healing. OSU – ref P2013-205-03

Publications

Original Articles

1. Roy S, Santra S, Das A, Dixith S, Sinha M, Ghatak S, Ghosh N, Banerjee P, Khanna S, Mathew-Steiner S, Das Ghatak P, Blackstone B, Powell HM, Bergdall V, Wozniak DJ, Sen CK. Staphylococcus aureus Biofilm Infection Compromises Wound Healing by Causing Deficiencies in Granulation Tissue Collagen. Ann Surg. 2020. 271(6):1174-1185. PMID: 30614873

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11. Elgharably H, Ganesh K, Dickerson J, Khanna S, Abas M, Ghatak PD, Dixit S, Bergdall V, Roy S, Sen CK. A modified collagen gel dressing promotes angiogenesis in a preclinical swine model of chronic ischemic wounds. Wound Repair Regen. 2014;22(6):720-9. PMID: 25224310
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Book Chapter

1. Ghatak PD, Sen CK. Antioxidant Additives in Food Preservation and Human Health In: Food Toxicology. Ed. Bagchi D, CRC Press/Taylor & Francis, Chapter 16. Academic Press.